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Full Length Research Paper

Frequency of extended spectrum beta-lactamase producing *Klebsiella pneumoniae* isolated from blood in Vojvodina, Serbia during 2009 to 2010

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The prevalence of extended-spectrum beta-lactamases (ESBL) varies between countries. The aim of this study was to determine the prevalence of ESBL producing *Klebsiella pneumoniae* originating from blood in the Vojvodina Province, Serbia. The study was conducted at Institute of Public Health of Vojvodina in Novi Sad, Serbia, in a two year period from January 2009 to December 2010. A total of 11894 blood samples from patients admitted to the Center were obtained for culture. Seventy-seven isolates of *K. pneumoniae* were reported. Antibiotic susceptibility testing and detection of ESBL production were carried out according to Clinical and Laboratory Standards Institute (CLSI) criteria. The ESBL phenotype was detected in 16 (53.3%) and 32 (68.1%) isolates in 2009 and 2010, respectively. Resistance to piperacillin/tazobactam increased from 43.7% in 2009 to 75% in 2010 while for amoxicillin/clavulanic acid decreased from 75 to 71.9% in the same period. Amikacin was more effective than gentamicin. More than 80% of isolates were resistant to cotrimoxazole and ciprofloxacin. All ESBL-producing isolates tested were sensitive to carbapenems. This study shows a very high prevalence of ESBL-producing *K. pneumoniae* among hospitalized patients with bloodstream infections and suggests that amikacin should be considered as synergistic antibiotic for the treatment of these infections together with other antimicrobial drugs.

Key words: *Klebsiella pneumoniae*, blood culture, antimicrobial resistance, extended spectrum beta lactamase.

INTRODUCTION

Klebsiella species, member of Enterobacteriaceae family is widely distributed in soil, water and the intestinal tract of humans and animals. Among *Klebsiella* species, *Klebsiella pneumoniae* (*K. pneumoniae*) is an important opportunistic nosocomial pathogen causing several diseases including pneumonia, wound and urinary tract infections, bacteriemia and meningitis. Immunocompromised patients, patients with severe underlying disease as well as those submitted to invasive diagnostic procedures and who stayed in the intensive care unit are the most susceptible to these infections. Risk factors for colonization and infection are recent surgery, central venous catheters, tracheotomy, parenteral nutrition, and uncontrolled use of broad spectrum antibiotics (third generation cephalosporins, fluoroquinolones, carbapenems). Reservoirs of infection are intestinal and respiratory tract of hospitalized patients and hospital staff, and the way of transmissions are the hands of the staff or contaminated medical equipment and fluids (Shayanfar et al., 2004; Kang et al., 2004; Abbott, 2003). Due to irrational use of antibiotics, *K. pneumoniae* isolated from

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Abbrevations: ESBL, Extended spectrum beta-lactamase; **MDR,** multidrug resistant; **KPC,** *Klebsiella pneumoniae* carbapenemase; **NDM-1,** New Delhi metallo-beta lactamase - 1; **CLSI,** clinical laboratory standards institute, **ATCC,** American type culture collection; **SPSS,** statistical package for the social sciences, **ICU** - intensive care unit.

blood culture is mostly multidrug resistant (MDR) to three or more antibiotic classes often producing beta-lactamases (Kang et al., 2004). The problem of a great concern is the emergence of *K. pneumoniae* strains producing extended spectrum beta-lactamases (ESBLs). ESBLs are plasmid mediated beta-lactamases capable of efficiently hydrolyzing all beta-lactam antibiotics including 3rd generation cephalosporins and monobactams, but not carbapenems (Mehrgan and Rahabar, 2008).

The first occurrence of ESBL producing organism was observed in France and Germany in 1983, and thereafter around the world (Paterson and Bonomo, 2005; Gagliotti et al., 2008). Until now more than 70 ESBLs have been described and most of them are members of TEM-1, TEM-2 and SHV-1 family of enzymes (Shayanfar et al., 2010; Behrooozi et al., 2010). During the past decade, rapid and massive spread of CTX-M-type ESBLs has been described. Now these enzymes are the most prevalent ESBLs in Enterobacteriaceae in Europe and in other areas of the World (Dallenne et al., 2010). The presence of ESBL-producing isolates is an indicator of the selective pressure of 3rd generation of cephalosporins which are extensively used in hospitals. Blood stream infections caused by ESBL producing bacteria were associated with longer, more costly hospital stays and high mortality rates (Ambretti et al., 2010).

The plasmid encoding ESBLs frequently also encodes the resistance to other classes of antimicrobials including fluoroquinolones and aminoglycosides leaving a few therapeutic options available for treatment (Abhilash et al., 2010; Eurosurveillance, 2010). Carbapenems that are stable to most of the prevalent beta-lactamases including ESBLs have become the treatment of choice for infection due to the ESBL producing strains. The first strain of *K. pneumoniae* producing plasmid-determined enzymes for inhibition of carbapenems was reported in 1996. They were serine carbapenemase KPC (*K. pneumoniae* carbapenemase). The majority of KPC harboring *K. pneumoniae* has been collected in the northeastern USA, they quickly spread and often appear in Israel and Greece, with outbreaks or isolated cases in hospitals in other European countries (Marschall et al., 2009; Woodford et al., 2004; Giakkoupi et al., 2009; Samuelsen et al., 2009; Grundmann et al., 2011).

Since 2008, Enterobacteriaceae producing New Delhi metallo-beta lactamase -1 (NDM-1) have been imported repeatedly into Europe from the Indian subcontinent particularly into the United Kingdom, but also to Austria, Belgium, France, Germany, The Nederlands, Norway and Sweden. Most of them were resistant to all antibiotics, except polymyxins, tigecycline and occasionally certain aminoglycosides. NDM-1 producers are mainly *K. pneumoniae* (Grundmann et al., 2011). It is necessary to investigate the frequency of ESBL and KPC positive strains in hospitals in order to formulate the policy of empirical therapy in high risk units where infections due to resistant organisms are much higher. The aim of this study was to determine prevalence of ESBL producing *K. pneumoniae* originating from blood in Vojvodina Province.

MATERIALS AND METHODS

Study design and specimen types

The study was conducted at the Center for Microbiology of Institute of Public Health of Vojvodina, Serbia. Samples of blood cultures from adult patients hospitalized on the various wards of Clinical center of Vojvodina Province were collected from January 2009 to December 2010. A total of 77 non-duplicate strains of *K. pneumoniae* were isolated. During the two year study period, 11894 blood samples were processed: 5753 samples in 2009 and 6141 in 2010.

Microbiological methods

All blood samples were routinely cultured in adult blood culture bottles (BioMérieux, Marcyl' Etoile, France) using standard precautions and processed by semi-automated blood culture system (BacT /Alert, BioMérieux, Marcyl' Etoile, France). The positive samples were inoculated onto blood agar and MacConkey agar plates (HiMedia, India) that were incubated aerobically 24 h at 37°C. Isolation and identification of the causative organisms were performed by standard microbiological methods.

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was tested by standard disk-diffusion method on Mueller-Hinton agar (HiMedia, India) using antibiotic discs BioRad, USA. The performance and interpretation were based on the recommendations of Clinical Laboratory Standards Institute (CLSI, 2009, 2010). The following antimicrobial agents were tested: ampicillin, cefalotin, ceftriaxone, ceftazidime, amoxicillin/clavulanic acid, piperacillin/tazobactam, meropenem, imipenem, ertapenem, gentamicin, amikacin, cotrimoxazole and ciprofloxacin. *Escherichia coli* American Type Culture Collection (ATCC) 25922 and *Staphylococcus aureus* ATCC 25923 were used for quality controls.

Detection of extended spectrum beta lactamases (ESBL) production

The initial screening and phenotypic confirmatory test was recommended by CLSI (CLSI, 2009, 2010). In this test a disc of amoxicillin/clavulanic acid was placed in center of the Petri plate already inoculated with the test microorganism while cefotaxime, ceftazidime and ceftriaxone discs were placed at a distance of 20 to 25 mm (center to center) from the amoxicillin-clavulanic acid disc on the same plate. After incubation of 18 to 24 h at 37°C zones of inhibition around the 3rd generation cephalosporin discs were observed. The microorganism was labeled as ESBL positive if the zone of inhibition around one or more cephalosporin discs was extended on the side nearest to the amoxicillin/clavulanic acid. The double-disk synergy test was used as the phenotypic confirmatory test where the test bacteria were grown on Mueller-Hinton agar and discs of cefotaxime and ceftazidime separately and each of these in combination with clavulanic acid were placed on the surface of plate with inoculated bacteria.

The microorganism was considered as ESBL positive isolate if

Table 1. *In vitro* resistance to antimicrobials of ESBL-positive and ESBL-negative *K. pneumoniae* strains isolated from blood in 2009.

Antibiotic	ESBL + (No = 16)		ESBL - (No = 14)	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
Ampicillin	0 (0)	16 (100)	0 (0)	14 (100)
Cefazolin	0 (0)	16 (100)	7 (50)	7 (50)
Ceftriaxone	0 (0)	16 (100)	11 (78.6)	3 (21.4)
Ceftazidime	0 (0)	16 (100)	11 (78.6)	3 (21.4)
Piperacillin/tazobactam	9 (56.3)	7 (43.7)	13 (92.9)	1 (7.1)
Amoxicillin/clavulanic acid	4 (25)	12 (75)	10 (71.4)	4 (28.6)
Imipenem	16 (100)	0 (0)	14 (100)	0 (0)
Meropenem	16 (100)	0 (0)	14 (100)	0 (0)
Ertapenem	16 (100)	0 (0)	14 (100)	0 (0)
Gentamicin	4 (25)	12 (75)	14 (100)	0 (0)
Amikacin	9 (56.3)	7 (43.7)	14 (100)	0 (0)
Cotrimoxazole	1 (6.3)	15 (93.7)	(78.6)	3 (21.4)
Ciprofloxacin	3 (18.7)	13 (81.3)	(78.6)	3 (21.4)

there was the difference of ≥ 5 mm between the zone of inhibition of a single antibiotic and in disc with combination of antibiotic and clavulanic acid (CLSI, 2009, 2010).

Statistical analysis

Laboratory data were entered into a database using SPSS (statistical package for the social sciences) for Windows. The χ^2 test was used to compare variables. All differences in which the probability of the null hypothesis was $p<0.05$ were considered significant.

RESULTS

K. pneumoniae was the 3rd leading isolated microorganism and the 2nd one among Gram-negative isolates out of the total 883. The frequency of isolation of *K. pneumoniae* was 7.5% (30 of 398 isolates) in 2009 and 9.7% (47of 485 isolates) in 2010. There was a high frequency of ESBL-producing *K. pneumoniae* in both years: 53.3% (16 out of 30) and 68.1% (32 out of 47) in 2009 and 2010 respectively. Despite the increase of incidence, the difference was not statistically significant. Resistance to piperacillin/tazobactam increased from 43.7 to 75% from 2009 to 2010, while for amoxicillin/clavulanic acid decreased from 75 to 71.9% in the same period. Among ESBL-producing strains, a total of 43 isolates (89.6%) were multidrug resistant (MDR). The most common MDR pattern was resistance to beta-lactams, aminoglycosides, cotrimoxazole and fluoroquinolones. Resistance to all antibiotics tested except carbapenems was higher in the ESBL-producing strains compared to the non-ESBL-producing bacteria. The most effective antibiotics for ESBL-producing strains were carbapenems which included imipenem, meropenem and ertapenem. Carbapenems had 100% activity in both years of investigation. Resistance to antimicrobial drugs during two years period is given in Tables 1 and 2. ESBL-producing *K. pneumoniae* were most commonly isolated in intensive care unit of the clinical center. The finding of ESBL-producing isolates in each ward of the Centre is shown in Table 3. The age of patients included in this study during 2009 to 2010 ranged from 20 to above 60 years. For the purposes of the study, patients were divided in different age groups: group I - patients from 20 to 39 years (n = 19, 24.7%), group II - patients from 40 to 59 years of age (n = 25, 32.5%) and group III patients above 60 years of age (n = 33, 42.8%). There was a significant difference between the first and the third age group of patients with bacteriemia due to ESBL producing and non-ESBL producing strains (p value < 0.05).

A total of 66.2% of the patients were males. According to the gender, a higher proportion of patients with ESBL-producing strains were found in the group of males (p value < 0.05) (Table 4).

DISCUSSION

The frequency of ESBL-producing isolates varies according to countries, region or even hospitals and is rapidly changing over time (Kader and Kumar, 2005). During the past decade, ESBL-producing *K. pneumoniae* has emerged as serious pathogen in hospitals worldwide. Recent studies revealed that patients with septicemia caused by ESBL producing organisms had significantly higher fatality rate than those with non-ESBL isolates (Mahrgan and Rahabar, 2008). In recent years, incidence of ESBL-producing *K. pneumoniae* is increased in many of the European countries such as Bulgaria, Greece, Croatia, Czech Republic, Italy and Hungary

Table 2. *In vitro* resistance to antimicrobials of ESBL-positive and ESBL-negative *K. pneumoniae* strains isolated from blood in 2010.

Antibiotic	ESBL + (No = 32)		ESBL - (No = 15)	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
Ampicillin	0 (0)	32 (100)	0 (0)	15 (100)
Cefazolin	0 (0)	32 (100)	11 (73.3)	4 (26.7)
Ceftriaxone	0 (0)	32 (100)	11 (73.3)	4 (26.7)
Ceftazidime	0 (0)	32 (100)	11 (73.3)	4 (26.7)
Piperacillin/tazobactam	8(25)	24(75)	11 (73.3)	4 (26.7)
Amoxicillin/clavulanic acid	9 (28.1)	23 (71.9)	11 (73.3)	4 (26.7)
Imipenem	32(100)	0 (0)	15 (100)	0 (0)
Meropenem	32(100)	0 (0)	15 (100)	0 (0)
Ertapenem	32(100)	0 (0)	15 (100)	0 (0)
Gentamicin	14 (43.7)	18 (56.3)	12 (80)	3 (20)
Amikacin	16 (50)	16 (50)	12 (80)	3 (20)
Cotrimoxazole	4(25)	28 (87.5)	10 (66.7)	5 (33.3)
Ciprofloxacin	2 (6.3)	30 (93.7)	11 (73.3)	4 (26.7)

Table 3. ESBL-positive and ESBL-negative *K. pneumoniae* isolates based on ward of admission.

Ward	2009		2010		Total	
	ESBL+(%)	ESBL-(%)	ESBL+(%)	ESBL-(%)	ESBL+(%)	ESBL-(%)
Internal	6 (7.8)	4 (5.2)	6 (7.8)	4 (5.2)	12 (15.6)	8 (10.4)
Surgery	4 (5.2)	1 (1.3)	6 (7.8)	2 (2.6)	10 (13)	3 (3.9)
Intensive care unit (ICU)	5 (6.5)	2 (2.6)	14 (18.2)	5 (6.5)	19 (24.6)	7 (9.1)
Obstetrics and gynecology	0 (0)	1 (1.3)	1 (1.3)	0 (0)	1 (1.3)	1 (1.3)
Other	1 (1.3)	6 (7.8)	5 (6.5)	4 (5.2)	6 (7.8)	10 (13)
Total	16 (20.8)	14 (18.2)	32 (41.6)	15 (19.5)	77 (100)	

Table 4. Demographic characteristics of patients according to ESBL producing *Klebsiella pneumoniae* bloodstream infection during 2009 to 2010.

Variables	ESBL + (%)	ESBL – (%)	Total (%)
Sex			
Male	32 (66.7)	19 (65.5)	51 (66.2)
Female	16 (33.3)	10 (34.5)	26 (33.8)
Age group			
20-39	13 (27.1)	6 (20.7)	19 (24.7)
40-59	14 (29.2)	11 (37.9)	25 (32.5)
> 60	21 (43.7)	12 (41.4)	33 (42.8)

(Eurosurveillance, 2010). In this study, *K. pneumoniae* was found to be the third most common etiological agent of bloodstream infections. Similar data are found by Al-Hasan et al. (2010a, b). The most prevalent ESBL-producing *K. pneumoniae* were isolated from patients in ICU ward of the Hospital which is in accordance with Paterson et al. (2003) and Ferrandez et al. (2011). In this study, the incidence of ESBL-producing *K. pneumoniae* isolated from blood was 53.3% in 2009 and 68.1% in 2010. Despite the increase of incidence, this difference was not statistically significant (p value > 0.05). ESBL prevalence varies in different countries. In some European countries, frequency of ESBL-producing *K. pneumoniae* is below 5% (Switzerland, Finland, Iceland,

Malta, Norway and Sweden) (Eurosurveillance, 2010). The ESBLs prevalence rate in this study was much higher than those reported in Iran (6.25%, Ramazanzadeh), Korea (15.9%, Kang), Thailand (23.7%, Musikatavorn), and in European countries [Slovenia (26%), Hungary (35%), Poland (37%), Italy (38%), Bosnia and Herzegovina (44%), Czech Republic (48%)] (Kang et al., 2004; Ramazanzadeh, 2010; Musikatavorn et al., 2011; Eurosurveillance, 2010).

Similar findings have been reported from some neighboring countries such as Bulgaria (73%), Greece (66%), Croatia (54%) (Eurosurveillance, 2009) and also by Abhilash who found that 72.4% isolates of *K. pneumoniae* were ESBL-producing in India (Abhilash et al., 2010). In a Turkish study by Serefhanoglu nearly 61% of blood culture isolates were ESBL-producing (Serefhanoglu et al., 2009) and the rate of 51.8% of ESBL-producing *K. pneumoniae* in bloodstream infection was found by Marra in the Brazilian hospital (Marra et al., 2006). Resistance to other antimicrobial drugs among ESBL-producing *K. pneumonia* was commonly found. Resistance to gentamicin was higher than amikacin. Resistance to cotrimoxazole and ciprofloxacin was higher than 80%. In this study, statistically significant increase of the resistance to piperacillin/tazobactam in 2010, compared to the results in 2009 was found (p value < 0.05). The increase of the resistance to amikacin and ciprofloxacin in 2010 was not significant (p value > 0.05). The susceptibility to non-beta-lactam agents in the ESBL-positive group was significantly lower than in ESBL-negatives. Resistance to carbapenem was not found in this study. KPC-positive bacteria were present in 1.3% of bacteremi an episode in a study by Marschall (Marschall et al., 2009). Presence of carbapenem resistant *K. pneumoniae* in bloodstream infection also confirms Mouloudi from Greece (Mouloudi et al., 2010). Carbapenem resistance is still rare in most countries. Seven European countries reported from 1 to 5% resistant strains (Bosnia and Herzegovina, Italy, Latvia, Norway, Portugal, Turkey and United Kingdom), but in three countries carbapenem resistance is considerably higher: Cyprus (10%), Israel (19%) and Greece (37%) (Eurosurveillance, 2010).

Some European authors reported emerging infections with NDM-1 producing strains (Eurosurveillance, 2010). Carbapenems are widely used for the treatment of infections caused by multidrug resistant gram-negative bacteria that produce extended-spectrum beta-lactamases. Several investigators have concluded that initial treatment of bloodstream infections caused by ESBL–producing strains with non-carbapenem agents may be associated with higher mortality than treatment with a carbapenem agent (Ambretti et al., 2010). In conclusion, our study shows a very high prevalence of ESBL-producing *K. pneumoniae* among hospitalized patients with bloodstream infections. Very high rates of resistance to all tested classes of antimicrobials except
carbapenems we noted. These data suggest that amikacin should be considered as synergistic antibiotic for the treatment of these infections, together with other anti-microbial drug. The study also highlights the need for the continual monitoring of antimicrobial susceptibility patterns of important bacterial pathogens so that rational antibiotic polices could be formulated. Further drug resistance surveillance is necessary in our hospitals as well as molecular characterization of ESBL isolates.

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